redhot diagnostics

PROVIDER OF CE-CERTIFIED LC-MS/MS DIAGNOSTIC KITS

INSTRUCTIONS FOR USE

FOR THE IN VITRO DETERMINATION OF PETH 16:0/18:1 IN BLOOD

CE

CE-IVD label according to European Directive 98/79/EC

Content

Phosphatidylethanol, PEth, LC-MS/MS Kit

Art.no. 50-2002, 200 analyzes including column.

Art. No. 50-2001, 200 analyzes, replacement it

US Pat. 9499572, 9784701

EP 2992334

1. INTENDED USE

For the in vitro determination of Phosphatidylethanol (PEth) 16:0/18:1 in blood

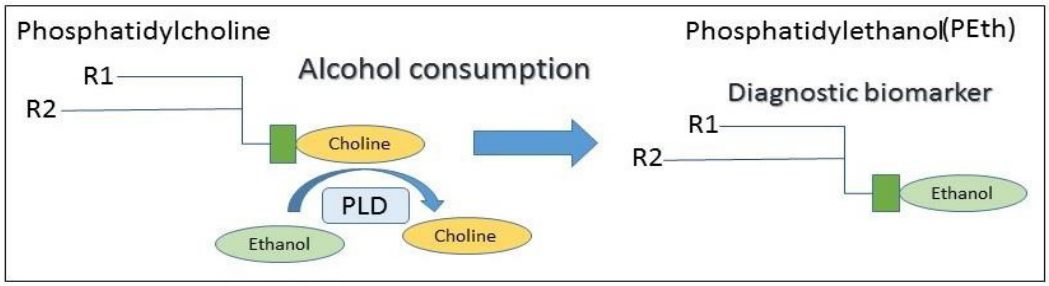
The described LC-MS/MS application is intended for the quantitative determination of phosphatidylethanol (PEth) in blood.

For in vitro diagnostics use.

2. INTRODUCTION

Phosphatidylethanol (PEth) in blood is a biomarker for previous alcohol consumption.

PEth is an unnatural phospholipid formed only in the presence of ethanol, giving a theoretical diagnostic specificity of 100% as a biomarker for alcohol consumption. The principle for in-vivo generation of PEth depends on rearrangement of phosphatidylcholine in presence of phospholipase D. PEth-16:0/18:1 is the most abundant individual form of PEth-homologues and is used in quantitative LC-MS/MS analysis of blood from patients to estimate the level of alcohol consumption. There is a direct correlation between alcohol consumption and the levels of PEth in blood.



PEth has been shown to be a more sensitive indicator of alcohol consumption than other markers. The half-life of PEth in circulation is three to eight days, which means that PEth can be detected up to 3 weeks after ethanol has been cleared from the body.

In a blood test the PEth concentration can very accurately be quantified by LC-MS/MS analysis.

The test developed by redhot diagnostics is robust with high specificity and sensitivity.

3. PRINCIPLES OF THE PROCEDURE

Phosphatidylethanol is extracted from blood by addition of 150 µL of extraction

solution, containing an internal standard (deuterated phosphatidylethanol), to 20 µL of blood. After thorough mixing, the tube is centrifuged, and an aliquot of the supernatant is injected in the LC-MS system. The components are separated on a column (art.no. 52-1000) using a binary gradient. The effluent is monitored with electrospray ionization mass spectrometry using multiple reaction monitoring (MRM) to follow the respective characteristic transitions for PEth and the internal standard. The ratio between the chromatographic peak areas for PEth and the internal standard are used to quantify the concentration of PEth in the samples.

4. WARNING AND PRECAUTIONS

Materials included in this kit should not be used past the expiration date on the kit label.

Reagents or substrates included in this kit should not be mixed or substituted with reagents or substrates from other kits.

PEth is sticky (may cause carry over) and it is important to choose efficient washing solutions and always inject at least a couple of blanks after a high standard sample level (§20).

5. HEALTH AND SAFETY PRECAUTIONS

Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to discard residues in accordance with laboratory regulations.

6. KIT CONTENTS

Art. no. 50-2001, 200 determinations, including column

Art. no. 50-2002, 200 determinations, replacement kit

	Component	Quantity
CAL	Calibrator in blood 0, 0.05, 0.1, 0.4, 0.8 and 1.6 µM	6 x 0.3 mL
IS	D_5-PEth 16:0/18:1, 4.5 nmol	0.5 mL
EXT	Extraction solution	45 mL
QC	In blood 0.3 µM	0.5 mL
TUN	Tuning solution	1 mL
COL	Column	1 pcs.

7. STORAGE CONDITIONS

The reagents should be stored at +2-8^{o}C

The Calibrator when diluted in whole blood should be stored frozen at -20°C

8. MATERIALS REQUIRED BUT NOT SUPPLIED

- LC-MS/MS-Equipment
- Column (replacement kit)
- Mobile phase A
- Mobile phase B
- Vortex
- Pipettes
- Centrifuge
- Vials

9. STARTUP – OPTIMIZATION OF PARAMETERS FOR THE ANALYTES

Use the included PEth tuning solution (TUN) to find the exact transitions for the MRM traces when setting up the kit for the first time.

PEth ionize and fragment easily and depending on instrument and parameters, the MS response might be too high for the electron multiplicator. If so, decrease the injection volume and/or dilute the samples.

Check the accuracy of the mass scales after annual preventive maintenance of the mass spectrometer, and after all other manipulations which can affect the accuracy of the mass scales.

10. PREPARATION OF REAGENTS

Blood samples (collected in EDTA vials) are suitable for the assay, either fresh or frozen blood samples can be used with the assay.

Allow samples and reagents to reach room temperature before use.

Important! Centrifuge the ampules containing calibrator (CAL) and internal standard (IS) briefly before opening the ampule (2000 RCF, 2 min).

11. PREPARATION OF EXTRACTION SOLUTION (EXT)

Centrifuge the ampule (IS) containing internal standard D_5-PEth 16:0/18:1 (2000 RCF, 2 min), open the ampule and add 0.5 ml Ext. sol., transfer the solution in its entirety to the Ext. sol. bottle, repeat 2 times to quantitatively transfer the internal standard to the Ext. sol. bottle. The final concentration of D_5- PEth 16:0/18:1 internal standard in Ext. sol. is 0.1

12. CALIBRATOR CURVE

The calibrator curve is ready to use. See sample preparation § 14

13. PREPARATION OF MOBILE PHASES

Mobile phase A

5 mM ammonium acetate in water	Preparation of 1 000 mL
Ammonium acetate	1 mL 5 M ammonium acetate
Milli-Q water	999 mL

Mobile phase B

10% 2-propanol in 90% methanol	Preparation of 1 000 mL
Methanol	900 mL
2-propanol	100 mL

14. SAMPLE PREPARATION

1. To 20 µL blood, add 150 µL Ext sol., vortex thoroughly for 2 x 5 sec.
2. Centrifuge the tubes at approximately 16400 RCF at 10°C for 10 min.
3. Transfer 120 µL of the supernatant to an autosampler vial, place the vial in the autosampler, inject 5 µL of the sample into the instrument.

15. CHROMATOGRAPHIC CONDITIONS LC-MS/MS METHOD

Listed as an example for Sciex API5500.

Instrument	Sciex API5500
Ionization	Electrospray
Scan Type	MRM
Polarity	ESI-
Curtain Gas	10
Collision Gas	8
Ion Spray Voltage (kV)	-4500
Temperature	500
Ion Source Gas 1	40
Ion Source Gas 2	40
DP (declustering potential)	150 – 220
CE (collision energy)	38 - 40

Transition used (Peth): 701.5 > 281.2
Transition used (D_5-Peth, IS): 706.5 > 281.2

16. GRADIENT

Flow rate: 0.4 mL/min

Analysis time: 7.5 min

Time [min]	MOP A [%]	MOP B [%]
0-1.0	50	50
1.0-3.0	13	87
3.0-3.5	0	100
3.5-5.5	0	100
5.5-5.6	50	50
5.6-6.0	50	50

17. CALCULATION

For each calibrator concentration, the peak area of the analyte is divided by the corresponding peak area of the internal standard. These ratios are plotted against the calibrator concentrations to calculate the calibrator curve equation, which is used to determine the PEth concentration of the sample. First order linear regression weighted by 1/x is preferred.

18. EXAMPLES OF CHROMATOGRAMS

Molecule	Monoisotopic mass
PEth 16:0/18:1	702.5
PEth-d5 16:0/18:1	707.5

Two different MRM transition fragments are possible to use (255 and 281). The best choice depends on instrument and parameters.

The PEth tuning solution will simplify finding which of the fragment to choose and exact decimals in the m/z values for each MS/MS instrument.

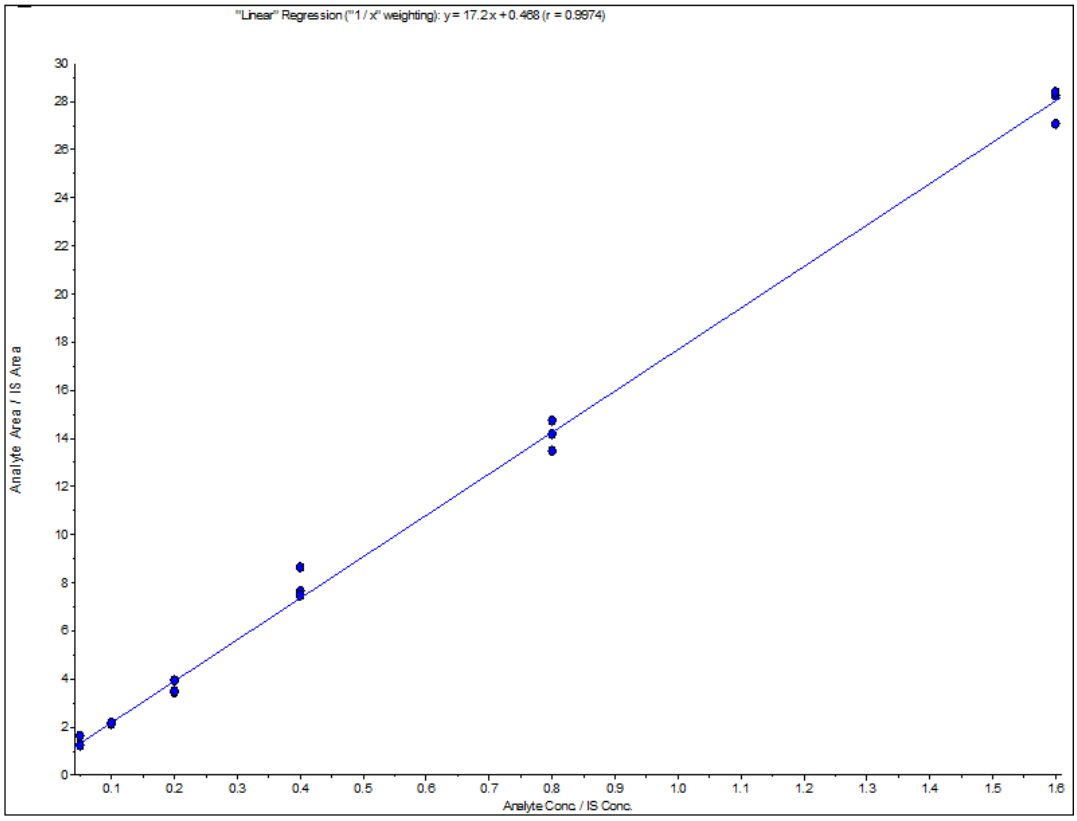


Figure 2. Calibration curve based on chromatograms from 701 -> 281

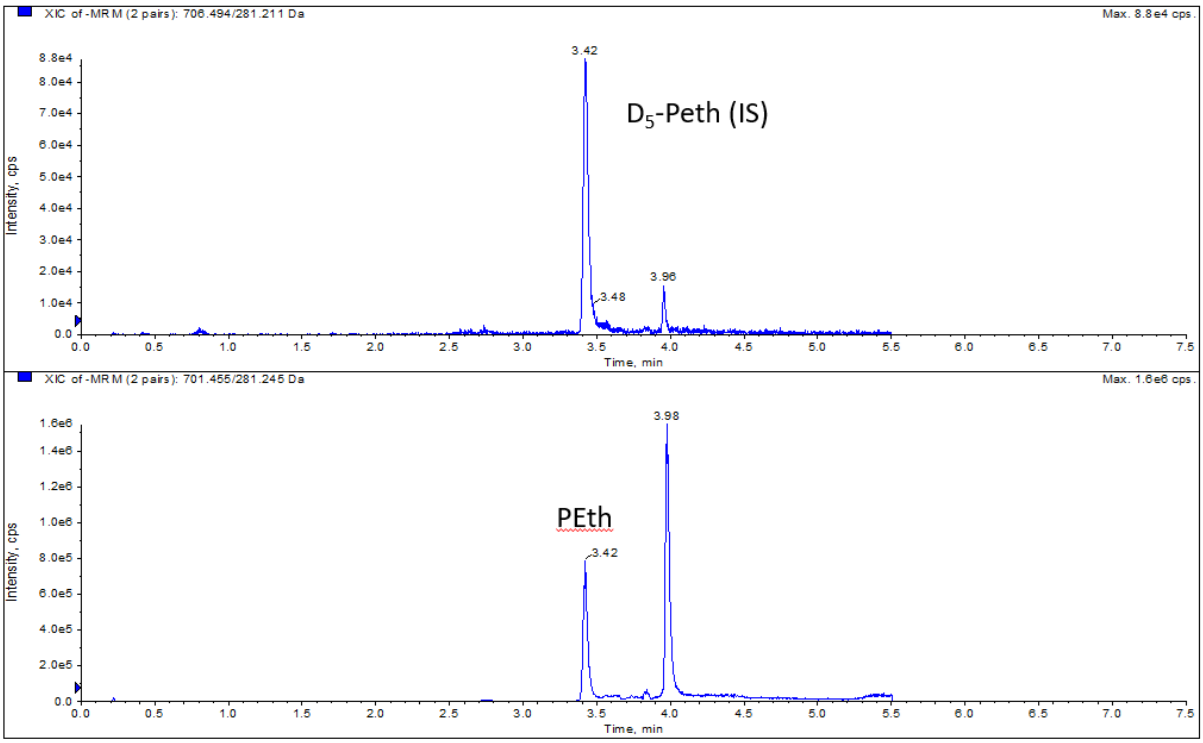


Figure 3. Control sample at 0.5 µM

19. QUALITY CONTROL

Control samples should be analyzed together with each batch of samples. Results generated from the analysis of control samples should be evaluated by statistical methods to ensure that the method shows accurate results. It is recommended to monitor the peak areas of the internal standard for all batches. The peak area should be consistent, and any inconsistency or systematic decrease of the internal standard can indicate interference, carryover effects and/or hardware related issues, such as contaminated column or ion source. Individual outliers can indicate issues with the sample or the preparation of the sample.

20. WASH SOLUTIONS:

Due to the sticky properties of PEth we recommend that the autosampler is washed with a mixture of 2-propanol, methanol, and ammonium acetate.

21. PERFORMANCE CHARACTERISTICS

Detection level

0.05 µM

Measuring range

0.05 – 1.6 µM

Samples over 1.6 µM should be diluted and analyzed again.

Reproducibility

Sample	PEth [µmol/L]	Intra Assay CV (%) (n=5)	Inter Assay CV (%) (n=5)
QC Low	0.25	<7.3	4.3 (n=3)
QC Mid	0.5	<12	11 (n=3)
QC High	1.0	<4.8	5.6

22. CE LABELING

The PEth kit is CE marked according to the EC in vitro diagnostic directive 98/79/EC.

23. REFERENCES:

1. Kechagias S, Dernroth DN, Blomgren A, Hansson T, Isaksson A, Walther L, Kronstrand R, Kågedal B, Nystrom FH. Phosphatidylethanol Compared with Other Blood Tests as a Biomarker of Moderate Alcohol Consumption in Healthy Volunteers: A Prospective

Randomized Study. Alcohol Alcohol. 2015 Jul;50(4):399-406.
2. Gustavsson L, Alling C. Formation of phosphatidylethanol in rat brain by phospholipase D. Biochem Biophys Res Com 1987, 142(3):958-63.
3. Helander A. and Zheng Y. Molecular Species of the Alcohol Biomarker Phosphatidylethanol in Human Blood Measured by LC-MS. Clinical Chemistry 2009, 55(7):1395-1405.
4. Gnann H, Engelmann C, Skopp G, Winkler M, Auwärter V, Dresen S, Ferreirós N, Wurst FM, Weinmann W. Identification of 48 homologues of phosphatidylethanol in blood by LC- ESI-MS/MS. Anal Bioanal Chem 2010, 396(7):2415-23.
5. Furey A, Moriarty M, Bane V, Kinsella B, Lehane M. Ion suppression; a critical review on causes, evaluation, prevention and applications. Talanta 2013, 15(115):104-22.
6. Viel G, Boscolo-Berto R, Cecchettto G, Fais P, Nalesso A, Ferrara SD. Phosphatidylethanol in blood as a marker of chronic alcohol use: A systematic review and meta-analysis. Int.J.Mol.Sci 2012, 13, 14788-14812
7. Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices